THE PHYSIOLOGY OF THE POLLEN OF TRIFOLIUM PRATENSE

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 175

J. N. MARTIN

(WITH ONE FIGURE)

The investigation of red clover pollen was begun with the hope that a thorough knowledge of its physiology, in conjunction with the history of the embryo sac, might help to overcome the uncertainty of clover seed production. The investigation was started at the University of Chicago during the summer term of 1912 and continued at Ames during the autumn of the same year. Many points demand further investigation, but the author thought it well to publish at this time, since the work cannot be resumed until the next growing season. The work has to do with three questions: conditions necessary for the germination of pollen; the stigma as a stimulative and directive factor in tube development; and relative potency of the pollen in self and cross-pollination.

Historical

HANSGIRG (8) and LIDFORSS (9, 11) succeeded in germinating the pollen of many species in tap water or moist air. The pollen of *Trifolium hybridum* germinated in moist air, but the pollen of *T. pratense* burst. RITTINGHAUS (5) found that the pollen of a large number of species would germinate in cane sugar solutions. The optimum concentrations for the different species varied from 20 to 40 per cent. MAX PFUNDT (14) showed that 20 to 50 per cent concentrations were required for the pollen of some grasses. KNY (3) found that the pollen of *Aesculus Hippocastanum*, *Lilium bulbiferum*, *Robinia Pseudo-Acacia*, *Lathyrus tuberosus*, and *Pisum sativum* germinated better when gelatin was added to the cane sugar solution. MANGIN (4) increased the germination in some species by adding either agar or gelatin to the sugar medium. JOST (12) found the germination of the pollen of some species of grasses to depend entirely upon the water supply. This he

controlled by germinating the pollen on parchment paper soaked in distilled water and properly dried on filter paper. The pollen of some composites would germinate on the parchment paper only after it had been soaked in sugar solution and then suitably dried. ELFVING (**2**) was unable to germinate the pollen of *Ornithogalum Ecklonii* and some species of grasses except on the stigma. Glycerin, potassium chlorate, and sodium carbonate added to sugar solutions had no effect. HANS MOLISCH (**7**) found that 0.01–0.05 per cent calcium malate or 0.01 per cent malic acid added to the sugar solution would cause the pollen of *Azalea* and *Rhododendron* to germinate. Saltpeter, asparagin, citric acid, and tartaric acid had a slight stimulative effect. LIDFORSS (**9**) increased the percentage of germination in some species of *Erica* and *Menziesia* by the addition of a small percentage of citric acid. VAN TIEGHEM (**1**) obtained better germination in some species by adding ammonium bitartrate to the medium. SANDSTEN (**16**) found that tomato pollen required a slightly acid medium. BURCK (**12**) observed that the pollen of certain species of *Mussaenda* would germinate in distilled water, but only when a portion of the stigma or levulose was added. Levulose could not be replaced by other sugars. TISCHLER (**15**) was able to germinate the sterile pollen common in *Solanum rostratum*, in some of the Commelinaceae, Melastomaceae, Pontederiaceae, Liliaceae, Lythraceae, and in the genus *Cassia* of the Leguminosae, by adding diastase to the sugar solution. According to LIDFORSS (**9**), the presence of a small percentage of calcium or potassium salts or a lack of aeration will prevent germination in many species. BURCK (**12**) found that levulose inhibited germination in *Pavetta* and *Antirrhinum* and caused bursting in *Murraya exotica*. The work of MOLISCH (**6, 7**) showed that the direction of pollen tubes in some species is due to carbohydrates, and in other species to negative aerotropism. LIDFORSS (**10**) found that proteins attract the pollen tubes in some species. In the species investigated by KNY (**3**) gravity and light had no directive influence on the pollen tubes. Some of the earlier botanists, and more recently LINDHARD (**17**), and the work carried on by PAMMEL and COE, which is not yet published, have shown that the pollen of *Trifolium pratense* is impotent on its own stigma.

Description of the pollen

The pollen grains mature very early in the history of the flower, the stamens and pistil being about 0.25 mm. in length and the integuments barely appearing on the ovules when the mother cells divide. When the embryo sac is mature the pollen grains are binucleate. They are almost globular when turgid, with a little flattening around the germ pores, and have an average size of 44.5 $\times 43\,\mu$. The pollen is plasmolyzed when shed from the anther, and one diameter is much shortened by an infolding of the wall. The average dimensions in this condition are $26 \times 48\,\mu$.

CONTENT.—Pollen treated with chloral hydrate and iodized potassium iodide gave no starch reaction. The immediate bursting of the pollen in these solutions permits good exposure of the contents and makes observations easy. Treated with Sudan III, the numerous small particles giving the pollen content its granular appearance gave a definite fat reaction. This fat exists in the form of a fine emulsion.

OSMOTIC PRESSURE.—In determining the osmotic pressure, sucrose solutions were used, since the pollen seems less permeable to this sugar. Volume-normal (mol. wt. dissolved to a liter volume of solution) solutions were used in watch glasses, which were sealed to prevent evaporation and left on the laboratory table. The results are given in table I.

TABLE I

Time	1.5 volume-normal	2 volume-normal	2.33 volume-normal	2.5 volume-normal	2.66 volume-normal
10 m....	Turgid	Turgid	Plasmolyzed	Plasmolyzed	Plasmolyzed
20 m....	"	"	"	"	"
30 m....	"	"	"	"	"
1 hr....	"	"	"	"	"
7 hrs...	"	"	5 per cent turgid	"	"
20 hrs...	"	"	10 per cent "	"	"
48 hrs...	"	"	20 per cent "		

The table shows that a 2.33 volume-normal solution is a little weak, provided the pollen grains are not permeable to the solution; but 2.33 volume-normal is nearer the proper strength than 2.5. If 2.33 volume-normal is changed to weight-normal (mol. wt.

dissolved in a liter of water) and calculated for 25° C. according to data and formulae given by RENNER (**18**), it gives an osmotic pressure of 163.5 atmospheres. This takes no account of the excessive increase of pressure over concentration between 4.13 and 4.65 weight-normal (2.33 volume-normal). The pollen was permeable to saturated solutions of KNO_3 and NaCl, and these salts could not be used for determining osmotic pressure.

Germination of pollen

The pollen of *Trifolium pratense* bursts almost instantly when dropped into water, so any suitable medium must control water absorption. Small amounts of sugar solutions made up in double distilled water by the volume-normal method were used in the ordinary watch glasses, carefully cleansed. The flowers were collected between 9 A.M. and 3 P.M. and the pollen from those well open, but still fresh, was used. The dishes, sealed with glass plates and vaseline, were left on the laboratory table and observations were made about every 30 minutes during the three or four-hour test period. Table II shows the effects of different sugar solutions on the pollen of *Trifolium pratense*, *T. hybridum*, and *T. repens*.

Decoctions of the stigmas alone or in distilled water, as well as those made by grinding the stigmas in the sucrose and levulose solutions given in table II, gave no germination in *Trifolium pratense;* 0.000075, 0.000375, and 0.075 volume-normal solutions of malic acid, as well as equal concentrations of calcium malate, added to the sugar solutions gave increased bursting in *T. pratense* and reduced the percentage of germination in *T. hybridum* and *T. repens*. A 0.000277 volume-normal of HCl or a 0.00056 volume-normal of butyric acid used with the sugar solutions had little effect. The butyric acid gave a little better germination in *T. hybridum* and *T. repens* in sucrose solutions above 0.731 volume-normal. Stronger solutions of either acid increased bursting and cut down germination. Sugar solutions containing agar or gelatin allowed less bursting, and 2 grams to 5 grams of gelatin added to a 0.731 volume-normal solution of sucrose gave the best medium for the pollen of *T. hybridum* and *T. repens*. Pollen of *T. pratense* run in sugar solutions under increased pressure of oxygen and

TABLE II

SHOWING THE EFFECTS OF DIFFERENT SUGAR SOLUTIONS ON THE POLLEN OF *Trifolium pratense*, *T. hybridum*, AND *T. repens*

Solution	Volume-normal	*T. pratense*	*T. hybridum*	*T. repens*
Double distilled H_2O		Immediate bursting	Immediate bursting	Immediate bursting
Sucrose	0.1462	Bursting	Bursting	Bursting
"	0.2824	"	"	"
"	0.4386	"	Bursting 25; germination	Bursting 25; germination
"	0.5848	About 50 burst; no germination	About 80 per cent germination	About 80 per cent germination
"	0.731	Turgid	Good germination	Good germination
"	0.8772	"	" "	" "
"	1.0233	"	" "	" "
"	1.1695	"	Plasmolysis	Plasmolysis
"	1.3217	"	"	"
"	1.4619	"	"	"
Levulose	0.833	Bursting	Some germination and much bursting	Some germination and much bursting
"	1.1108	About 50 per cent bursting	Fair germination; no bursting	Fair germination; no bursting
"	1.3888	Turgid; no bursting	About 50 per cent germination; no bursting	About 50 per cent germination; no bursting
"	1.666 2.2216 2.499 2.7776	Turgid; no bursting	About 50 per cent germination; no bursting	About 50 per cent germination; no bursting
Dextrose	1.1108	Bursting	Bursting	Bursting
"	1.3888	"	Feeble germination; some bursting	Feeble germination; some bursting
"	1.666	"	Feeble germination; some bursting	Feeble germination; some bursting
"	1.9333	"	No bursting; 25 per cent germination	No bursting; 25 per cent germination
"	2.2216 2.499 2.7776	"	No bursting; 25 per cent germination	No bursting; 25 per cent germination
Maltose	0.5848	"	Bursting	Bursting
"	0.731	Some bursting	Feeble germination; some bursting	Feeble germination; some bursting
"	0.8772	Bursting	Feeble germination; some bursting	Feeble germination; some bursting
"	1.0233	"	No bursting; 25 per cent germination	No bursting; 25 per cent germination

TABLE II—*Continued*

Solution	Volume-normal	*T. pratense*	*T. hybridum*	*T. repens*
Maltose...	1.1695 1.3217 1.4619	Bursting	No bursting; 25 per cent germination	No bursting; 25 per cent germination
Percentages of maltose and dextrose mixed gave about the same results				
Lactose...	0.5848	Bursting	Bursting	Bursting
" ...	0.731	"	50 per cent germination; some bursting	50 per cent germination; some bursting
" ...	0.8772	"	50 per cent germination; some bursting	50 per cent germination; some bursting
" ...	1.0233 1.1695 1.3217 1.4619	"	50 per cent germination; no bursting	50 per cent germination; no bursting
Arabinose	1.3333	"	Bursting	Bursting
"	1.6555	"	50 per cent germination; some bursting	50 per cent germination; some bursting
"	1.9999	"	50 per cent germination; no bursting	50 per cent germination; no bursting

carbon dioxide showed that the supply of these gases was not the limiting factor. Small dishes made by cutting off glass shells about one-half inch from the bottom and containing the pollen in very shallow depths of sucrose and levulose solutions, which did not permit bursting, were placed in wide-mouthed bottles and attached to oxygen and carbon dioxide tanks. No germination resulted from a three-hour exposure to an increased pressure of these gases. In table III the results obtained with other media are given.

Results obtained by use of parchment paper and animal membrane with pollen of Trifolium pratense and T. hybridum

Small squares of parchment paper were soaked in distilled water and in 0.5848, 0.731, and 0.8772 volume-normal sucrose solutions and then dried on filter paper until surplus moisture was removed, mounted on slides, and after application of pollen placed

under bell jars on the laboratory table. Parchment paper proved unsatisfactory because its opaqueness and fibrous character made observation difficult; so after several sets were run with no germination, hog bladder was substituted. After a few trials good

TABLE III

SHOWING THE EFFECTS OF VARIOUS SOLUTIONS ON THE POLLEN OF *Trifolium pratense*

Solutions			
Sucrose 0.731+KNO_3 0.00006.	Bursting		
Sucrose 0.731+KNO_3 0.0002..	About 25 per cent bursting	75 per cent turgid	No germination
Sucrose 0.731+KNO_3 0.0006..	About 25 per cent bursting	75 per cent turgid	" "
Sucrose 0.731+KNO_3 0.002..	Very little bursting	Turgid	" "
Sucrose 0.731+KNO_3 0.005...	No bursting	"	" "
Sucrose 0.731+galactose 1.3888 + a trace of asparagin.....	Very little bursting	"	" "
Sucrose 0.731+asparagin 0.0375.....	Bursting		
Sucrose 0.731+dextrose 1.3888 +KNO_3 0.0005.....	Little bursting	25 per cent or more turgid	" "
Sucrose 0.731+0.0075 asparagin.....	About 50 per cent bursting	50 per cent turgid	" "
Sucrose 0.8772+dextrose 1.666 +asparagin 0.075.....	Bursting	.....	.....
Sucrose 1.0233+asparagin 0.15	"	.....	.....
Sucrose 0.8772+galactose 1.9333+asparagin 0.187....	"	.....	.....
Sucrose 0.8772+asparagin 0.0015.....	No bursting	Turgid	" "
Sucrose 0.8772+asparagin 0.00075.....	" "	"	" "
Sucrose 0.8772+asparagin 0.000375.....	" "	"	" "
Sucrose 0.8772+glycerin oleic acid.....	" "	"	" "
Sucrose 0.8772+palmitic acid..	" "	"	" "
Sucrose 0.8772+lipase.....	" "	"	" "
Levulose 1.666+lipase.....	" "	"	" "
Lecithin+H_2O as a thick paste.	" "	"	" "
Sucrose+lecithin as a thick paste.....	" "	"	" "
Levulose+lecithin as a thick paste.....	" "	"	" "
Sucrose 0.8772+diastase.....	" "	"	" "

germination was obtained on the bladder. It was found that germination was very closely connected with the amount of water in the membrane, and it was not easy to dry the membrane so as always to secure germination. Membranes soaked in

0.731 and 0.8772 volume-normal solutions of sucrose or in 1.3888 and 1.666 volume-normal solutions of levulose and properly dried gave as good results as those soaked in distilled water. This shows that these sugars have no toxic effect on the pollen. The efficiency of the membrane did not depend upon the fats or salts contained, for pieces extracted 5 days in alcohol and ether in a Soxhlet's extraction apparatus or boiled for 16 hours in changes of distilled water did not lose their efficiency, although their physical properties were so changed that the requisite amount of soaking and drying had to be found again by experimentation. Another series of trials with parchment paper showed it to be as effective as the bladder and that the previous failure was due to insufficient drying. In these tests most attention was given to the pollen of *T. pratense*, although the pollen of *T. hybridum* was investigated sufficiently to discover that it germinated readily on the membrane and that its germination would permit more variation in the water content of the membrane than the pollen of *T. pratense*.

The nature of the germination of the pollen of *T. pratense* on the membrane needs some discussion. Germination was not uniform. On some portions of the membrane the percentage of germination was high, while on other portions there was no germination. These different regions were usually quite definitely marked off, and the germination in a region was usually good or none at all. This lack of uniformity was mainly due to a difference of texture, composition, or thickness of these regions. A difference in these properties would make a difference in the amount of water supplied to the pollen in the different regions. Some variation no doubt exists between pollen from different anthers and between pollen grains from the same anther in respect to the water supply requisite for germination. But in mounting the pollen, the keel was sprung with a scalpel, and as the pollen was thrown from the anthers, it was collected on the instrument and then spread on the membrane. By this method of collecting and mounting, the pollen was well mixed, and the variation of the pollen would not account for all of the lack of uniformity in germination.

The percentage of germination determined by taking into account all the pollen on a membrane when any germination occurred

varied from almost zero to 96 on different membranes. Still, on membranes with the low percentage of germination, the percentage of germination was high in the region where it occurred. In table IV are given the results obtained on a small piece of bladder that gave fairly uniform germination.

TABLE IV

Total number of pollen grains	Number of germinations	Percentage of germination
107	87	81+
135	110	81+
209	183	87+
176	170	96+
126	101	80+
153	144	94+

The time required for germination at room temperature was 8–10 minutes. This agrees with SANDSTEN'S (**16**) report on *T. hybridum* and *T. repens*.

The lengths of tubes produced were various. The maximum length of tubes measured was about 15 times the diameter of the pollen grain. The lengths of the majority ranged from 6 to 15 times the diameter of the pollen. It is probable that much longer tubes would have been produced if the water delivery of the membrane had remained constant.

The results obtained with the membrane and parchment paper showed that the water supply was at least the important factor if not the only factor in determining germination.

An attempt was made to secure the proper water supply by means of sugar solutions. Sucrose solutions with a difference of 0.0877 volume-normal and ranging from 0.731 to 2.2 volume-normal were used. The only germination obtained in these solutions was less than 0.5 per cent in 1.7 volume-normal. In accounting for this failure, three things should be considered: (1) the range of water supply permitting germination may be so small that it was missed by these concentrations; (2) the supply of oxygen and carbon dioxide might have been limiting factors since the higher concentrations were greater than those run under the increased pressure of these gases; (3) the condition for germina-

tion may be a certain ratio between the amount of water taken up and transpiration.

An effort was made to reduce this required water supply to some definite expression by running tests on bladder suspended over different concentrations of H_2SO_4. Gram-molecular solutions were placed in large, wide-mouthed bottles fitted with rubber corks. A glass tube about 10 inches in length was run through the rubber cork and the membrane suspended from a cork fitted over the lower end of the glass tube. With the upper end of the tube corked, the apparatus was left in the required temperature 48 hours to secure moisture equilibrium between liquid and membrane. If stored longer than 48 hours fungi gave trouble. The pollen was collected on the end of a glass rod and deposited on the membrane by running the rod through the glass tube. This method prevented interchange between outside and inclosed air. The percentages of moisture were approximated from data given in LANDOLT-BÖRNSTEIN (**19**). The humidity at the pressure of saturation over pure water was considered 100 per cent, and moisture for each temperature used and the percentages over the solutions are based on the 100 per cent. The results of two series run are given in full to show variation and the others are summarized (tables V–VII).

TABLE V

TEMPERATURE 35° C.

Gram molecular of H_2SO_4	Relative percent. of moisture	No. of pollen grains	Percent. of germinations	Condition
1	95.5	1201	0.88	Turgid
0.7	96.5	2187	17.4	Some bursting
0.5	97.2	1801	29.9	Little bursting
0.3	98.6	2209	10.0	20 per cent bursting
Pure H_2O	Some less than 100	2736	24.4	30 per cent bursting

TEMPERATURE 20° C.

1	95.5	1120	0	Turgid
0.7	96.5	1396	0.21	"
0.5	97.2	1590	0.12	"
0.3	98.6	1340	1.34	Little bursting

TABLE VI

RELATIVE PERCENTAGE OF MOISTURE 98; GRAM-MOL. 0.5 H_2SO_4; TEMPERATURE 35° C.

No. of sets run	No. of pollen grains	No. of germinations	Percentage of germinations	Condition
1st	208	0	0	Turgid
2d	200	0	0	"
3d	198	0	0	"
4th	205	125	60.9	"
5th	223	146	64.5	Some bursting
6th	181	5	2.7	Turgid
7th	208	0	0	"
8th	195	165	84.6	"
9th	183	98	53.5	"
Totals	1801	539	29.9	

TABLE VII

RELATIVE PERCENT. OF MOISTURE 99; GRAM-MOL. 0.3 H_2SO_4; TEMPERATURE 35° C.

No. of sets run	No. of pollen grains	No. of germinations	Percentage of germinations	Condition
1st	219	0	0	Turgid
2d	167	0	0	50 per cent bursting
3d	127	0	0	Turgid
4th	132	0	0	"
5th	253	50	19.7	"
6th	218	100	45.8	"
7th	310	10	32.2	Much bursting
8th	435	35	8+	" "
9th	348	26	7.5	" "
Totals	2209	221	10	

As seen from the tables, the percentages of germination in most of the sets run were low as compared with those obtained under bell jar on the laboratory table. This low percentage may be due to three things: (1) the membrane was not in equilibrium; (2) the amount of moisture required by different pollen grains for germination may vary so much that only a small percentage of germination can take place under a given moisture condition; (3) germination may be to some extent connected with transpiration. The marked variation in behavior between sets run over the same solution at the same temperature strongly suggests that moisture equilibrium had not been established within the apparatus. The

tables show that for these temperatures germination takes place only when the percentage of moisture is close to saturation.

The influence of the stigma upon the germination of the pollen and upon the direction of the pollen tubes

The stigma presents a very uneven surface due to the projection of the papillae. The exposed portion of the papillae has a rather heavy cutinized wall. Microchemical tests showed no sugar or starches present in the papillae, but an oily emulsion such as was found in the pollen.

Although decoctions of the stigmas had been tried without any positive results, it was thought worth while to try them in connection with the bladder. After the pollen had been spread on the prepared sections of bladder, stigmas from other plants were pressed down on these membranes with a scalpel, and then the sets were run under the bell jar on the laboratory table. These stigmas apparently exerted no influence on germination or on the direction of the pollen tubes. Often there was no germination around the stigma when there was good germination in other regions; and when there was germination around the stigma, germination just as good could be found in other places. The pollen tubes around the stigmas were grown in all directions, and pollen grains in contact with the stigma were found growing tubes at right angles to, and away from, the stigma. From these observations it appears that the stigma secretes nothing that has any effect on germination or the direction of the pollen tube. The behavior of the stigma in the experiments at least indicates that its function in the germination of the pollen is to regulate the water supply; and the nature of the pollen necessitates no other function. If this is the function of the stigma, and the water supply must be as delicately adjusted on the stigma as on the membrane to secure germination, then conditions which will modify the amount of water delivered by the stigma will have an effect on fertilization and hence on seed production. This may account for the usually poor seed production in the early part of the season, since there is usually more moisture in the ground at this time and more rain during the flowering period than occurs during the second crop. If germination depends

upon a certain balance between the amount of water taken up and transpiration, then a variation in the moisture of the atmosphere would have an effect on fertilization.

The comparative potency of pollen in self and cross-pollination

Pollen tubes can be traced through the stylar canal by mounting the pistils in a 30 per cent sucrose solution and flattening with a little pressure on the cover glass. The tubes have a denser and more granular content than the cells of the style and these features make it possible to trace them. Sufficient pressure causes the ovary to break just above the ovules and enables one to see the tubes in the ovary. Fig. 1 shows the upper portion of an ovary with exposed pollen tubes 55 hours after pollination. For self and cross-pollination vigorous field plants were selected, and flowers to be used were put under cover 2 or 3 days previous to opening and remained covered until collected for examination. Flowers were self-pollinated by simply springing the keel. In cross-pollination the keel was sprung, and the pollen collected from another plant was applied to the stigma with a scalpel. An examination of 30 flowers crossed showed the pollen tubes in the ovary 50 hours after pollination. Sections of ovaries killed 55 hours after crossing showed that the egg had enlarged for its first division

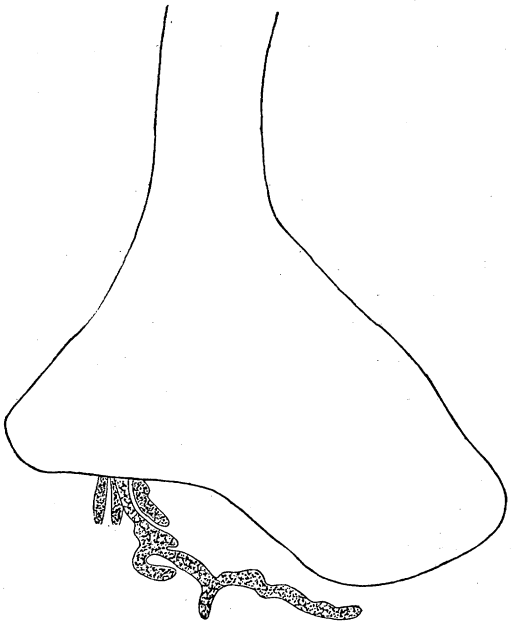

FIG. 1.—Camera drawing, showing the upper portion of an ovary teased off and the pollen tubes invading the ovary.

and that the endosperm cell had made one division; therefore, fertilization must take place about 50 hours after cross-pollination.

An examination of those flowers self-pollinated at the same time the others were crossed showed good germination on the stigma. The number of pollen grains germinating on their own stigma ranged from 3 to 25 in the 30 flowers. The tubes produced were all short, none exceeding 4 mm. Out of 20 self-pollinated flowers run 90 hours, one tube was found with a length of 7.25 mm.; the other tubes varied in length from a fraction of a millimeter up to 5 mm. Counting the average length of the style and stigma 11.5 mm., one is able to compare the rates of growth of pollen tubes in the two cases. The tubes in case of self-pollination look as vigorous as those in cross-pollination. Some abnormal behavior was observed. In one case the tubes were found wound about each other in the upper part of the stylar canal. In a few cases one of the longer tubes had turned back upon itself.

The question is now raised in case of self-pollination as to whether or not the tube can reach the ovary and effect fertilization. Field work on self-pollination shows that it rarely does, if ever.

Summary

The pollen of *Trifolium pratense* is physiologically different from that of *T. hybridum* and *T. repens* in respect to behavior toward sugar solutions.

The only function of the sugar solution in the case of the pollen of *T. hybridum* is the controlling of water supply.

The germination of the pollen of *T. pratense* is delicately adjusted to water absorption.

The results of the investigation show that the stigma produces no secretions which influence pollen tubes.

The nature of the pollen demands no other function of the stigma in its germination than the control of the water supply.

The pollen in self-pollination germinates readily on the stigma, but the tubes traverse the style much more slowly than in cross-pollination.

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IOWA STATE COLLEGE
AMES, IOWA

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